ZOOLOGICAL SCIENCE 9: 1017–1023 (1992)

Analysis of Sex Steroids in Feces of Giant Pandas

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ABSTRACT—Concentrations of testosterone (T) and estradiol-17β (E_2) in feces were measured in giant pandas (*Ailuropoda melenoleuca*). Feces were collected almost monthly from October 1987 to August 1988 and from June 1989 to July 1990, from captured pandas (a male and a female adult pandas and their two children) at Ueno Zoo, Tokyo. The elder child born in 1986 was identified later as the female and the younger one born in 1988 as the male. The concentrations of T in feces of the adult male was always higher than that in the female when compared in the same month. The concentration of E_2 in feces of the adults did not show clear difference between sexes. In the adult female, a high E_2 peak was observed in March of 1988, when it showed estrus and was artificially inseminated. No E_2 peak was detected in the spring of 1990, when it did not show clear estrus. Concentrations of T and E_2 in the children, especially in the younger child, were relatively low and variable. They showed no consistent seasonal changes.

These results suggest that the sexing of adult pandas is possible by comparing the concentration of T in feces. Peaks of the fecal steroid content seem to show roughly reproductive condition of an animal, suggesting a possibility that ovulation can be detected in the female panda by the fecal E_2 analysis.

INTRODUCTION

As well-known, the giant panda (*Ailuropoda melenoleuca*) is an endangered mammalian species distributed only in limited areas in China. Although its ecology has been thoroughly studied (see Schaller *et al.* [1]), much remains to be clarified. Especially, it is important to know reproductive physiology in wild individuals of this species. Analysis of sex steroid hormones in blood plasma is a popular and indispensable method for studying reproductive physiology of vertebrates. However, it is impossible or extremely difficult to collect blood samples from wild individuals of this species in the field or even from captive individuals in many cases. In captive individuals, analysis of urinary steroid can be an alternative, but it is practically difficult to collect urinary samples especially from infant pandas.

Attempts have been made to estimate gonadal endocrine activity of mammals and birds by analyzing sex steroids in feces [2–8]. This non-invasive method was recommended by Risler *et al.* [2] as a useful tool in ecophysiological studies and diagnosis of pregnancy in wild mammals or mammals from which collection of blood or urinary sample is difficult. Czekala and Lasley [5], and Stavy *et al.* [6] employed this method for sexing of monomorphoic bird species.

Motivated by the study of Risler *et al.* [2], the authors intended to study the relation between feal sex steroids and sex or reproductive condition in adult and infant giant pandas by measuring sex steroids in feces which were collected in different months from two infant giant pandas and their parents.

Accepted July 23, 1992
Received June 12, 1992
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MATERIALS AND METHODS

Animals

Two infant giant pandas and their parents reared in Ueno Zoo, Tokyo were used. The father (named Fei Fei) was born in 1967 in Sichuan (China), caputured in 1976 and brought to Ueno Zoo in November 1982. The mother (Huan Huan) was born in 1972 in Sichuan (China), captured in 1975 and brought to Ueno Zoo in January 1980. The infants (Tong Tong and You You) were born in June 1986 and June 1988, respectively, and have been reared in Ueno Zoo. It was unable to sex these infants at the time of their birth morphologically. Three to four years later, the elder and younger infants were identified as the female and the male, respectively, from their behavior and the difference in the length between the anus and urogenital opening.

Collection of feces

Fecal samples were collected from the floor of cages in which animals were kept once a month on 20th day of each month at around 5 am during the following two periods: the first from October 1987 to August 1988 and the second from June 1989 to August 1990. The feces were considered to be excreted between 9 pm and 5 am, since the floor of the cages used to be cleaned at 9 pm every day. One of fecal droppings or masses found in the floor was randomly selected for each animal at each collection time and frozen in a freezer at $-20^{\circ}C$ soon after collection and stored for a few months until steroid analysis. The mean weight $\pm$ standard deviation (SD) of the selected fecal dropping was 56.46 ± 20.22 g in the father, 52.44 ± 15.52 g in the mother, 54.39 ± 19.16 g in the elder infant and 49.54 ± 22.24 g in the younger infant.

Extraction

We followed the method of Risler *et al.* [2] with slight modification. Feces were partially thawed at the room temperature, and thick threads of bamboo contained were removed with forceps. Then, 30 to 100 g of the feces were homogenized in a grinder with 10 volumes of a mixture of ethanol and acetone (8:2). After adding about 1,000 cpm of tritiated steroids for determining the recovery rate, the homogenate was centrifuged at 3,000 rpm for 10 min at $4^{\circ}C$. The supernatant was filtered through a cellulose membrane filter (0.2 μm pore size, FR-20, Fuji Film Co. Ltd, Tokyo). After concentrating the volume to about 5 ml by centrifuging evaporation in vacuo, the volume was adjusted to 20 ml by adding ethanol and water (8:2). This solution was placed in a freezer at $-20^{\circ}C$ for 12 to 18 hr to precipitate fat. After centrifuging at 2,000 rpm at $-10^{\circ}C$ for 20 min, fat was removed. To the ethanolic supernatant, 5 volumes of petroleum ether was added. After vortexing, the petroleum ether layer was removed and discarded. The bottom layer was evaporated to 1 to 2 ml, and extracted two times with 5 volumes of ethyl ether. The combined ether phase was washed two times with 1 ml of 8% sodium bicarbonate solutions with pH 10 and 8, respectively, and then the ether phase was evaporated to dryness under nitrogen. The residue was dissolved in 0.5 ml of methanol and used as the sample for chromatography.

Chromatography

Estradiol-17β (E_2) was separated from testosterone and progestrone by the DEAE A-25 column chromatography according to the method of Risler *et al.* [2]. The DEAE A-25 sephadex was changed from the chloride form to the hydroxide form just before the column chromatography, and suspended in 100% methanol. Columns were made of Pasteur pipettes, and packed with 1.5 ml of the gel. After applying the sample in 0.5 ml methanol, the neutral steroids were eluted with 5 ml of methanol, and then the phenolic steroids with 5 ml of 0.1 M acetic acid in methanol. Each eluate was evaporated to dryness and dissolved in 0.5 ml of methanol.

The mean recovery $\pm$ SD through the whole course of the extraction and separation procedures was $73.6 \pm 21.3\%$ in E_2 and $65.9 \pm 17.0\%$ in testosterone.

Radioimmunoassay

Testosterone and E_2 in the chromatographic fractions were determined by radioimmunoassay using testosterone-3-(O-carboxymethyl) oximino-(2-[^{125}I]iodo-histamine) of Amersham, England,

respectively, as radioligands, and the rabbit anti-testosterone serum (HAC-AA61-02-RBP81) and the rabbit anti-estradiol serum (HAC-AA63-01-RBP7), respectively, provided by Prof. Katsumi Wakabayashi of Gunma University. The separation of free and bound steroids was performed with the second antibody method using a goat anti-rabbit gamma G serum provided by Prof. Katsumi Wakabayashi. The standard (12.2 pg to 6.25 ng of testosterone or E_2/0.05 ml) or an appropriately diluted unknown sample (0.05 ml) was preincubated with the corresponding first antiserum (0.05 ml) and buffer (0.1 ml) at 4°C for 24 hr.

Then, the mixture of the sample and antiserum was reacted with corresponding radioligand (10,000 cpm/0.05 ml) at 4°C for 48 hr. The incubation with the second antiserum (0.1 ml of the 200 times diluted serum) contained with 4% polyethylene glycol was performed at room temperature for 3 hr. The mean intra- and inter-assay coefficients of variation were 1.5 and 4.3%, respectively, in the testosterone assay. They were 2.9 and 7.87%, respectively, in the E_2 assay.

RESULTS

Testosterone and estradiol concentration in mature pandas

The testosterone concentration in feces of the parents varied widely among months, but the range of the variation was smaller, between 8 and 880 pg/g, in the mother than in the father, between 105 and 8,910 pg/g (Fig. 1). Especially, the fecal testosterone level in the mother was significantly lower than that in the father when compared in the same month ($P<0.01$ by the analysis of variance with two-way layout). In addition, seasonal changes in the testosterone level were similar between the two observation periods (one from June 1987 to August 1988 and the other from June of 1989 to July of 1990) in both the father and mother, although data of August and September in 1989 and April and May in 1990 were lacked accidentally in both individuals.

In the father, there were two peaks of testosterone, one in the autumn (October or earlier in 1987 and October in 1989) and the other in the spring or late winter (April in 1988 and February in 1990). Corresponding exactly to these spring peaks in the

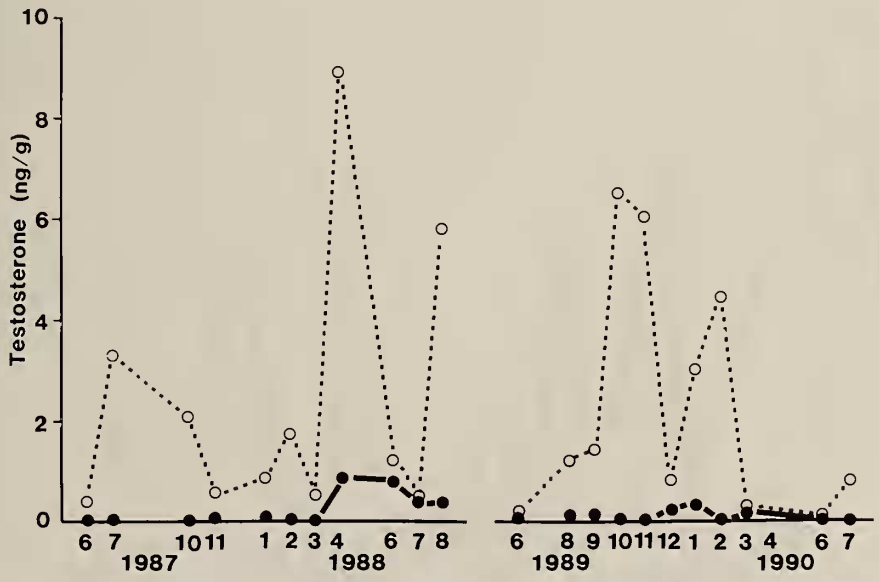

Fig. 1. Monthly changes of immunoreactive testosterone levels in feces of mature male panda (Fei Fei) (open circles and dotted line) and mature female panda (Huan Huan) (closed circles and solid line) from June of 1987 to August of 1988, and from June of 1989 to Jyly of 1990. Samples were collected in 20th of each month. Each point indicates the mean of duplicate determinations for each fecal dropping.

father, there were peaks of testosterone around the same time also in the mother. Supporting this, Kendall's rank correlation analysis revealed that there was a statistically significant positive correlation ($\tau=0.75$, $P<0.05$, n=9) in the fecal testosterone concentration between the father and the mother in the earlier half of the year (from January to June) but not significant correlation or even negative correlation ($\tau=-0.60$, $P>0.05$, n=10) in the variable in the later half of the year (from July to December).

The concentration of estradiol-17β (E_2) in feces as well as the concentration of testosterone varied widely among months in both the mother (near 0 to 700 pg/g) and the father (near 0 to 1,064 pg/g) (Fig. 2). However, unlike the testosterone concentration, the estradiol concentration did not differ clearly between the father and the mother ($P>0.1$ by the analysis of variance).

In the father, the E_2 level elevated conspicuously in January and February in both observation periods, showing the highest peaks in February (Fig. 2). Peaks of E_2 were also found in June or Jyly in both observation periods in the father. To confirm the parallelism of the seasonal changes in fecal E_2 between these two periods, the correlation analysis was applied. There was found a statistically significant positive correlation ($\tau=0.763$, $P<0.05$, n=7) in the estradiol concentration in the father between the two observation periods.

In the mother, the concentration of E_2 in feces had the highest peak of the year in November in both observation periods. High estradiol levels were also observed in June and July in both observation periods. Only in the first observation period, there was an additional high E_2 peak in March. The correlation of the estradiol concentration in the mother between the two observation periods was not statistically significant ($\tau=0.112$, $P>0.05$, n=7).

Testosterone and estradiol-17β concentrations in children

In the elder child, the concentration of testosterone in feces was low in the first observation period, having three peaks in October 1987, January 1988

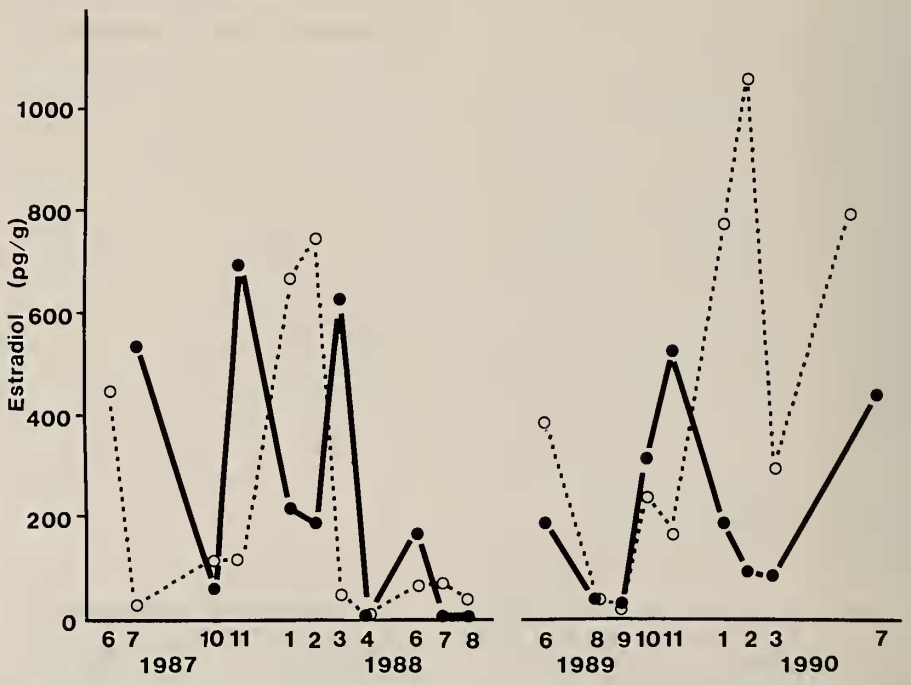

FIG. 2. Monthly changes of immunoreactive estradiol levels in feces of mature male panda (Fei Fei) (open circles and dotted line) and mature female panda (Huan Huan) (closed eireles and solid line) from June of 1987 to August of 1988, and from June of 1989 to Jyly of 1990. Samples were collected in 20th of each month. Each point indicates the mean of duplicate determinations for each fecal dropping.

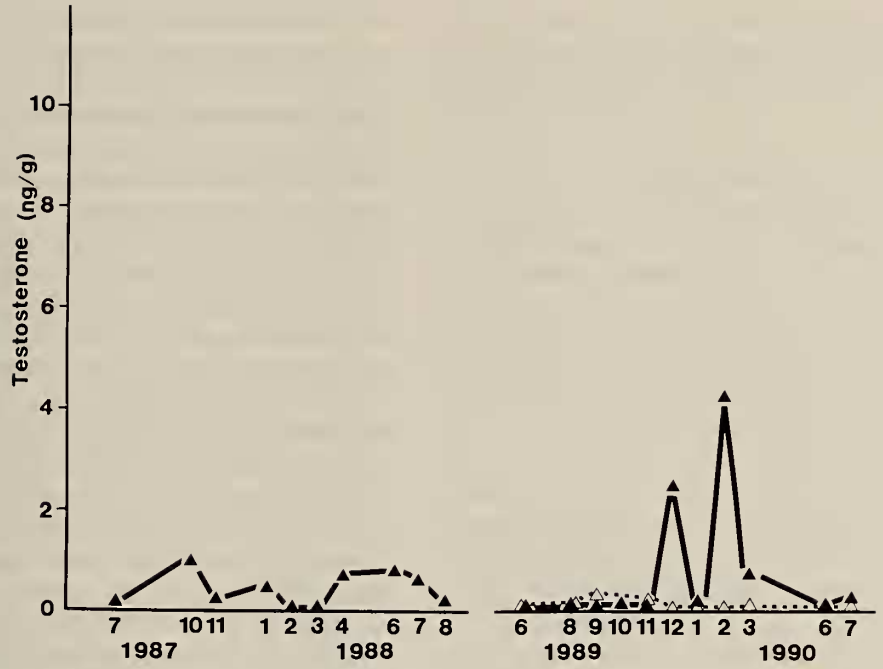

FIG. 3. Monthly changes of immunoreactive testosterone levels in feces of infant elder panda (Tong Tong) (closed triangles and solid line) and younger panda (You You) (open triangles and dotted line) from June of 1987 to August of 1988, and from June of 1989 to July of 1990. Samples were collected in 20th of each month. Each point indicates the mean of duplicate determinations for each fecal dropping.

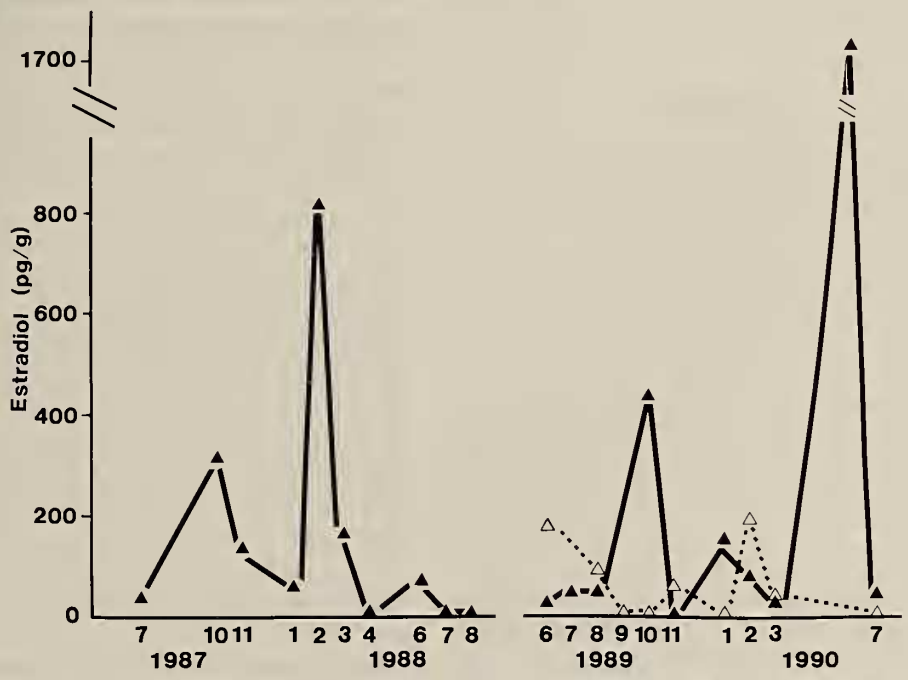

FIG. 4. Monthly changes of immunoreactive estradiol levels in feces of infant elder panda (Tong Tong) (closed triangles and solid line) and younger panda (You You) (open triangles and dotted line) from June of 1987 to August of 1988, and from June of 1989 to July of 1990. Samples were collected in 20th of each month. Each point indicates the mean of duplicate determinations for each fecal dropping.

and June 1988, respectively (Fig. 3). However, the range of the fluctuation was small, and the highest peak was about 1,000 pg/g. In the second observation period, the testosterone levels were still low except the two peaks, one higher than 2,000 pg/g in December 1989 and the other higher than 4,000 pg/g in February 1990 (Fig. 3). These basal and peak testosterone levels in the elder child were clearly lower than the respective levels in the father. The two peaks in the second observation period in the elder child were higher than the highest testosterone peak in the mother, but the levels in the other months in the elder child were similar to the level in the mother.

The concentration of E_2 in feces of the elder child had three peaks in each of the two observation periods (Fig. 4). Their locations (October, January or February and June) coincided approximately between the two observation periods, although their heights were different.

In the younger child, the concentration of testosterone in feces was extremely low, always less than 400 pg/g (Fig. 3). The concentrations of E_2 in feces of the younger child were low and had three peaks in November, February and presumably June (Fig. 4).

DISCUSSION

Validity of the method of fecal sex steroid analysis must be discussed first. Positive proof of validity of this method for estrogens including E_2 and progesterone was demonstrated first by Adlercreuz and Jarvenpaa [7] with human feces. They identified the steroids in the feces by gas chromatography and mass spectrometry. They also found that 85 to 90% of excreted estrogen occurs in unconjugated form and also that fecal estrogen and progesterone well reflect reproductive conditions of men and women. Most or a part of their results have been confirmed in *Macaca* by Risler *et al.* [2].

In addition, analysis of fecal E_2 and testosterone was successfully applied for sexing in three species of birds by Stavy *et al.* [6] and for estimation of gonadal endocrine activity in the Japanese quail [8], although feces of birds contain urine. Thus, there will be little doubt about the validity of fecal sex steroid analysis for assessing gonadal endocrine activity in mammals and birds.

It is obvious that feces of the father contain higher concentration of testosterone than feces of the mother. This difference can be due to either sexual difference or a coincident individual variation. The former possibility is supported by the following facts revealed in the present study: 1) the fecal testosterone showed a peak in the breeding period of this species, 2) similar seasonal changes were observed in the two observation periods, 3) the testosterone levels in the father were higher than those in the children, and 4) peaks of the testosterone level appeared simultaneously in the breeding season also in the mother, although the peak levels were lower in the mother. It is also noteworthy that there were additional increases in fecal testosterone in the autumn in the father. This fact reminds us the report that the giant panda breeds some times in the autumn [1]. Furthermore, in Ueno Zoo, artificial collection of spermatozoa became possible from October in the father, and they were available until May or June. Thus, the possibility of the difference in the testosterone concentration in feces between the mother and the father is obviously high, and hence the possibility of a coincident individual variation is low.

We have no proper explanation for the fact that the father excreted as large amounts of E_2 in feces as the mother. However, this is not so surprising, since Adlercreuz and Jarvenpaa [7] reported that men and postmenopausal women excreted similar mounts of estrogen in feces. The presence of the estradiol peaks in November in both of the two observation periods in the mother is favorable to the theory of the presence of the additional breeding period in the autumn. There was a high peak of estradiol in March of 1988, when the mother was artificially inseminated and pregnancy was induced. She gave birth of You You in June of the same year. However, there was no conspicuous estradiol peak in March in 1990, when artificial insemination was attempted again but no pregnancy was induced this time.

Fecal testosterone excreted by the elder child in the first observation period was as small in amount as that excreted by the mother. However, in the

second observation period, there were found two high peaks of fecal testosterone in December and February which were higher than the highest peak in the mother but lower than that in the father. However, unlike the father, the elder child did not show high autumnal increase in the fecal testosterone. Amounts of excreted testosterone in feces by the younger child were too low to discuss the seasonal excretion pattern.

The elder child showed a high fecal estradiol level in February 1988. This level is as high as the peak levels observed in the parents. In 1990, there was found an extremely high estratiol excretion in feces in the elder child in June. This level was higher than any of the highest levels observed in the parents. Interpretation of this estradiol level in June is difficult.

The younger child excreted small amounts of estradiol in feces in both 1989 and 1990. These amounts are similar to those excreted in months of 1987 by the elder child who is 2 years older.

In conclusion, this study suggested with a high possibility that we can sex adult giant pandas by analysis of fecal sex steroids, especially testosterone, although more examples are needed to conclude. It may be also possible to estimate gonadal endocrine activity of giant pandas indirectly by analyzing fecal sex steroids successively from the same individual. However, sexing of infant giant pandas by analysis of fecal sex steroids may be difficult.

ACKNOWLEDGMENTS

The authors thank to Mr. T. Hahn, University of Washington for his reviewing the manuscript and Prof. K. Wakabayashi for providing the antisera. This work was supported by grants of the Ueno Zoological graden, Waseda University and Ministry of Education, Science and Culture.

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